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(54) RECOMBINANT HUMAN-BASIC FIBROBLAST GROWTH FACTOR (RH-BFGF) AND PHARMACEUTICAL COMPOSITION COMPRISING RH-BFGF

(57) Provided are a mutated nucleic acid molecule of a recombinant human-basic fibroblast growth factor (rh-bFGF), a pharmaceutical composition of the rh-bFGF, and a use thereof.

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Description

Technical Field

[0001] The invention relates to the field of DNA recombination and biopharmaceuticals. More specifically, the invention relates to a mutated nucleic acid molecule encoding a recombinant human-basic fibroblast growth factor (rh-bFGF), a pharmaceutical composition comprising the rh-bFGF, and use of the pharmaceutical composition for treating dry eye.

Background Technique

[0002] Dry eye is a general term for various diseases caused by abnormalities in tear quality or kinetics caused by any cause, resulting in decreased tear film stability, and accompanied by eye discomfort and/or ocular surface tissue lesions, also known as keratoconjunctivitis sicca.

[0003] When various causes, such as reduce in the production of tear film liquid tear, abnormal mucus secretion and meibomian gland dysfunction, cause the absolute and relative lack of tear film components, abnormal distribution of tears on the ocular surface, and increase in tear evaporation, dry eye can be caused.

[0004] Common symptoms include dry eyes, easy fatigue, itchy eyes, foreign body sensation, burning sensation, thick secretions, fear of wind, photophobia, sensitivity to external stimuli; sometimes the eyes are too dry, the basic tears are insufficient, which in turns stimulates reflexive tear secretion, causing frequent lachrymation; in severe cases, the eyes will be red, swollen, keratinized, and the corneal epithelium will be broken with filaments adhered. This damage can cause keratoconjunctival lesions and affect vision in a long term.

[0005] Nowadays, the number of patients with dry eye is increasing. Currently, commercially available products such as sodium hyaluronate eye drops, polyvinyl alcohol eye drops, polyethylene glycol eye drops, etc. can alleviate dry eye symptoms, but can only act as artificial tears. The natural tear is complex in composition, and the integrity of the three-layer structure of the natural tear film is the basis for the implementation of its effective function, but the natural tear cannot be completely replaced with an artificial tear.

[0006] In addition, preservatives are currently contained in commercially available products, and long-term use of preservative-containing drugs may cause damage to the ocular surface and may cause ocular surface diseases to be more severe.

[0007] There is currently no product using bFGF for treating dry eye, and no bFGF derived from human has been reported for treating dry eye.

Summary of the Invention

[0008] In one aspect, the invention provides a mutated nucleic acid molecule encoding a recombinant human-basic fibroblast growth factor (rh-bFGF).

[0009] In one aspect, the invention provides a recombinant human-basic fibroblast growth factor (rh-bFGF) encoded by the mutated nucleic acid molecule.

[0010] In one aspect, the invention provides a pharmaceutical composition comprising the recombinant human-basic fibroblast growth factor (rh-bFGF) and at least one pharmaceutically acceptable excipient.

[0011] In one aspect, the invention provides a pharmaceutical composition comprising the recombinant human-basic fibroblast growth factor (rh-bFGF) and at least one stabilizer selected from the group consisting of glycine, histidine, arginine, Tween, heparin sodium or human serum albumin (HSA).

[0012] In one aspect, the pharmaceutical composition is free of preservatives.

[0013] In one aspect, the invention provides a method of treating dry eye, the method comprising administering to a patient in need thereof a therapeutically effective amount of the pharmaceutical composition.

Drawings

[0014]

Figure 1 is a diagram showing the structure of the pET-30a(+) plasmid.

Figure 2 shows the results of Western Blot of rh-bFGF.

Figure 3 shows the purity of rh-bFGF detected by high performance liquid chromatography.

Figure 4 shows the molecular mass spectrum of the rh-bFGF intact protein.

Figure 5 shows the cDNA sequence and amino acid sequence of the native human bFGF and the amino acid sequence of the mutated human bFGF.

Figure 6 shows a four-parameter fitting curve for the assay of the rh-bFGF *in vitro* activity, where C is the EC50 value.

Figure 7 shows the effect of different amino acids, including histidine, glycine and arginine, and Tween on the purity change of rh-bFGF under the hot stress condition, wherein 95%LINE refers to the quality standard for the purity of the rh-bFGF stock solution; and a purity of less than 95% for the stock solution means that the purity is unqualified.

Figure 8 shows the effect of HSA on the purity change of rh-bFGF under the hot stress condition.

Figure 9 shows the effect of different concentrations of the rh-bFGF eye drop on tear secretion in a dry eye model of alkali burned New Zealand rabbits. The symbol "*" or "**" indicates a significant difference ($p<0.05$ or $p<0.01$).

Figure 10 shows the effect of different concentrations of the rh-bFGF eye drop on the break-up time of the tear film in a dry eye model of alkali burned New Zealand rabbits. The symbol "*" or "**" indicates a significant difference ($p<0.05$ or $p<0.01$).

Detailed Description of the Invention

[0015] In one aspect, the inventors have for the first time developed a pharmaceutical composition comprising a recombinant human-basic fibroblast growth factor and one or more pharmaceutically acceptable excipients.

[0016] In one aspect, the invention provides a pharmaceutical composition comprising the recombinant human-basic fibroblast growth factor (rh-bFGF) and at least one stabilizer selected from the group consisting of glycine, histidine, arginine, Tween, heparin sodium or human serum albumin (HSA). In this regard, it has unexpectedly been found that the recombinant human-basic fibroblast growth factor and the stabilizer constitute a specific stable combination.

[0017] In one aspect, the pharmaceutical composition is free of preservatives.

[0018] In one aspect, the invention provides a mutated nucleic acid molecule encoding the recombinant human-basic fibroblast growth factor (rh-bFGF). In one aspect, the invention provides a mutated nucleic acid molecule encoding the recombinant human-basic fibroblast growth factor (rh-bFGF), which comprises a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3, or comprises a sequence having at least 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3. In one aspect, the invention provides a mutated nucleic acid molecule encoding the recombinant human-basic fibroblast growth factor (rh-bFGF), which has the sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

[0019] In one aspect, the invention provides a recombinant human-basic fibroblast growth factor (rh-bFGF) encoded by the mutated nucleic acid molecule.

[0020] In one aspect, the invention provides a pharmaceutical composition comprising the recombinant human-basic fibroblast growth factor (rh-bFGF) and at least one pharmaceutically acceptable excipient. In one aspect, the excipient includes, but is not limited to, a buffer system, a thickener, a stabilizer, a neutralizing agent, a humectant, and the like.

[0021] In one aspect, the present invention provides a pharmaceutical composition comprising the recombinant human-basic fibroblast growth factor (rh-bFGF) and at least one stabilizer selected from the group consisting of glycine, histidine, arginine, Tween, heparin sodium or human serum albumin (HSA).

[0022] In one aspect, the excipient comprises a buffer system. The buffer system includes, but is not limited to, sodium dihydrogen phosphate-disodium hydrogen phosphate, citric acid-disodium hydrogen phosphate or boric acid-borax. In one aspect, the buffer system is sodium dihydrogen phosphate-disodium hydrogen phosphate. In one aspect, the buffer system is citric acid-disodium hydrogen phosphate.

[0023] In one aspect, when sodium dihydrogen phosphate and disodium hydrogen phosphate are used as the buffer system, sodium dihydrogen phosphate and disodium hydrogen phosphate are present at concentrations of 0.25mg/mL-1.25mg/mL and 1.25mg/mL-3.75mg/mL, respectively.

[0024] In one aspect, when sodium dihydrogen phosphate and disodium hydrogen phosphate are used as the buffer system, sodium dihydrogen phosphate and disodium hydrogen phosphate are present at concentrations of 0.2-2.5 mg/mL and 0.5-5.0 mg/mL and 0.5-5.0 mg/mL, respectively.

[0025] In one aspect, when sodium dihydrogen phosphate and disodium hydrogen phosphate are used as the buffer system, sodium dihydrogen phosphate and disodium hydrogen phosphate are present at concentrations of 0.425mg/mL and 2.50mg/mL, respectively.

[0026] In one aspect, the excipient comprises a thickener. The thickener includes, but is not limited to, polyvinyl alcohol, sodium hyaluronate, methyl cellulose, hydroxymethyl cellulose, hydroxyethyl cellulose, hypromellose, poloxamer or carbomer thickener. In one aspect, the thickener is polyvinyl alcohol. In one aspect, the thickener is a carbomer. In one

aspect, the carbomer is such as a series of Carbomer 940, Carbomer 934, Carbomer 974, Carbomer 980, etc., preferably a Carbomer 980 series.

[0027] In one aspect, the thickener is typically present at a concentration of 0.1-20.0mg/mL. In one aspect, the thickener is typically present at a concentration of 5.0-15.0 mg/mL, such as 5.0 mg/mL, 6.0 mg/mL, 7.0 mg/mL, 8.0 mg/mL, 9.0 mg/mL, 10.0 mg/mL, 11.0 mg/mL, 12.0 mg/mL, 13.0 mg/mL, and 14.0 mg/mL.

[0028] In one aspect, when polyvinyl alcohol is used as the thickener, the amount of polyvinyl alcohol in the composition is typically 5.0 mg/mL, 6.0 mg/mL, 7.0 mg/mL, 8.0 mg/mL, 9.0 mg/mL, 10.0 mg/mL.

[0029] In one aspect, when carbomer is used as the thickener, the amount of carbomer in the composition is typically 5.0 mg/mL, 6.0 mg/mL, 7.0 mg/mL, 8.0 mg/mL, 9.0 mg/mL, 10.0 mg/mL.

[0030] In one aspect, the excipient comprises a stabilizer. Such stabilizer includes, but is not limited to, heparin sodium, human serum albumin (HSA), glycine, histidine, arginine, Tween, or a combination of two or more thereof. In one aspect, the stabilizer is heparin sodium and human serum albumin (HSA). In one aspect, the stabilizer is human serum albumin (HSA). In one aspect, the stabilizer is glycine. In one aspect, the stabilizer is histidine. In one aspect, the stabilizer is arginine. In one aspect, the stabilizer is Tween, such as Tween-20.

[0031] In one aspect, when heparin sodium and human serum albumin are used as the stabilizer, the amount of heparin sodium in the composition is typically 0.1-100 μg/mL, such as 0.25-75.0 μg/mL, such as 0.25-5.0 μg/mL, 25.0-75.0 μg/mL, such as 10 μg/mL, 15 μg/mL, 20 μg/mL, 25 μg/mL, 30 μg/mL, 35 μg/mL, 40 μg/mL, 45 μg/mL, 50 μg/mL, 55 μg/mL, 60 μg/mL, 70 μg/mL, or 75 μg/mL; the amount of human serum albumin in the composition is typically 0.01-10.0 mg/mL, such as 0.03-10.0 mg/mL, 0.025-0.375 mg/mL, 0.1-0.375 mg/mL, such as 0.1 mg/mL, 0.15 mg/mL, 0.2 mg/mL, 0.25 mg/mL or 0.3 mg/mL.

[0032] In one aspect, when human serum albumin is used as the stabilizer, the amount of human serum albumin in the composition is typically 0.01-10.0 mg/mL, such as 0.03-10.0 mg/mL.

[0033] In one aspect, when glycine, histidine, or arginine is used as the stabilizer, the amount of glycine, histidine, or arginine in the composition is typically 2%-5% (w/v), such as 2%, 3%, 4% or 5%.

[0034] In one aspect, the excipient comprises a neutralizing agent. The neutralizing agent includes, but is not limited to, triethanolamine, sodium hydroxide, potassium hydroxide, sodium carbonate, sodium hydrogen carbonate, potassium hydrogen carbonate or borax. In one aspect, the neutralizing agent is triethanolamine.

[0035] In one aspect, the neutralizing agent is typically present at a concentration of 1.25-12.5 mg/mL, such as 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL or 10 mg/mL. In one aspect, when triethanolamine is used as the neutralizing agent, the amount of triethanolamine in the composition is typically 5-12.5 mg/mL, such as 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL or 10 mg/mL.

[0036] In one aspect, the excipient comprises a humectant. The humectant includes, but is not limited to, glycerin, propylene glycol or a mixture thereof.

[0037] In one aspect, the humectant is typically present at a concentration of 0.1-50.0 mg/mL. In one aspect, when glycerol is used as the humectant, the amount of glycerin in the composition is typically 12.5-50.0 mg/mL, such as 12.5-25.0mg/mL, 25.0-50.0 mg/mL, such as 25.0mg/mL.

[0038] In one aspect, the composition is buffered to a pH of about 6.0-8.0, such as a pH of 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 or 8.0 or a pH defined by any range therebetween.

[0039] In one aspect, the invention provides a method of treating dry eye, the method comprising administering to a patient in need thereof a therapeutically effective amount of the pharmaceutical composition.

[0040] In one aspect, the invention provides the pharmaceutical composition for use in treating dry eye.

[0041] In one aspect, the invention provides use of the pharmaceutical composition for treating dry eye.

[0042] In one aspect, the invention also provides use of the composition in the manufacture of a medicament for treating dry eye.

[0043] In one aspect, the pharmaceutical composition of the invention can be administered by a variety of routes of administration, such as by ocular administration to a patient in need thereof, such as administration by conjunctiva.

[0044] The pharmaceutical composition of the invention can be administered in various dosage forms, for example, as a solution, a suspension, an emulsion, a microemulsion, a composite emulsion, a drop, a gel, a spray, an eye drop, an ophthalmic ointment, or an ophthalmic lotion and an injection.

[0045] In one aspect, the pharmaceutical composition of the invention is an ophthalmic pharmaceutical composition. In particular, the pharmaceutical composition of the invention is in the form of an eye drop or a gel.

[0046] An advantage of the pharmaceutical composition of the present invention is that the rh-bFGF and the stabilizer such as glycine, histidine, arginine, Tween, heparin sodium or human serum albumin (HSA) unexpectedly constitute a specific stable combination. In particular, glycine, histidine, arginine, Tween, heparin sodium or human serum albumin (HSA) significantly improves the stability of rh-bFGF. Furthermore, the humanized bFGF of the invention is immunologically safer than non-human derived bFGF.

[0047] The invention is further described with reference to the following non-limiting examples.

Example

Example 1. Nucleic acid mutation

[0048] The cDNA sequence (SEQ ID NO: 4) encoding the native human bFGF was mutated without changing the amino acid sequence of the protein, and three mutated cDNA sequences were designed and synthesized (mutated cDNA Sequence 1, Sequence 2 and Sequence 3 as showed by SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3, respectively):

Mutated cDNA Sequence 1 (SEQ ID NO: 1)

atg gct gct ggt tcg att acg acg ctg ccg gct ctg ccg gaa gat ggt ggt tca ggt gca ttt ccg ccg ggt cac ttt aag gat ccg aaa cgt ctg tat tgc aag aac ggc ggc ttt ttc ctg cgc att cat ccg gat ggc cgt gtc gac ggt gtg cgc gaa aaa agc gat ccg cac att aag ctg cag ctg caa gca gaa gaa cgt ggc gtg gtt agc atc aaa ggt gtt tgt gcg aac cgt tac ctg gcc atg aaa gaa gat ggc cgc ctg ctg gct agt aag tgc gtc acc gac gaa tgc ttt ttc ttt gaa cgt ctg gaa tcc aac aat tat aat acc tac cgt agc cgc aaa tat acg tct tgg tat gtg gcc ctg aaa cgc acg ggc cag tat aag ctg ggt tcc aaa acg ggt ccg ggt caa aaa gcc att ctg ttc ctg ccg atg tcc gca aaa tca taa

Mutated cDNA Sequence 2 (SEQ ID NO:2)

atg gct gct ggt tct atc acc acc ctg ccg gct ctg ccg gaa gac ggt ggt tct ggt gct ttc ccg ccg ggt cac ttc aaa gac ccg aaa cgt ctg tac tgc aaa aac ggt ggt ttc ttcctg cgt atc cac ccg gac ggt cgt gtt gac ggt gtt cgt gaa aaa tct gac ccg cac atc aaa ctg cag ctg cag gct gaa gaa cgt ggt gtt gtt tct atc aaa ggt gtt tgc gct aac cgt tac ctg gct atg aaa gaa gac ggt cgt ctg ctg gct tct aaa tgc gtt acc gac gaa tgc ttc ttc ttc gaa cgt ctg gaa tct aac aac tac aac acc tac cgt tct cgt aaa tac acc tct tgg tac gtt gct ctg aaa cgt acc ggt cag tac aaa ctg ggt tct aaa acc ggt ccg ggt cag aaa gct atc ctg ttc ctg ccg atg tct gct aaa tct taa

Mutated cDNA Sequence 3 (SEQ ID NO:3)

atg gca gcc ggt agc atc acc acc ctg ccg gcc ctg ccg gag gat ggc ggc agc ggc gcc ttc ccg ccg ggc cac ttc aag gac ccg aag cgt ctg tac tgc aaa aac ggt ggc ttc ttc ctg cgc atc cac ccg gac ggc cgt gtt gac ggt gtc cgt gag aag agc gac cct cac atc aag ctg caa ctg caa gca gaa gag cgt ggt gtt gtg tct atc aaa ggt gtg tgt gct aac cgt tac ctg gct atg aag gaa gat ggt cgt ctg ctg gct tct aaa tgt gtt acc gat gag tgt ttc ttt ttt gaa cgt ctg gaa tct aac aac tac aac act tac cgt tct cgt aaa tac acc tct tgg tat gtg gca ctg aaa cgt act ggt cag tat aaa ctg ggt tcc a aa acc ggt cct ggt cag aaa gct atc ctg ttt ctg cca atg tct gct aag agc taa

Example 2. Prokaryotic expression and protein characterization

2.1 Expression and purification

[0049] The expression vector used was pET-30a(+) (see Figure 1) and was purchased from Merk KgsA co. (Cat. No. 69909-3). The vector carries a T7 promoter, a T7 transcription initiation site, a His Tag, a coding sequence, an S Tag coding sequence, multiple cloning sites (MCSs), a T7 terminator, a lactose coding sequence, a kan resistance coding sequence, and a pBR322 replicon and a f1 replicon. The MCSs comprise the restriction sites Xho I, Not I, Eag I, HindIII,

Sal I , Sac I, EcoR I, BamH I, EcoR V, Nco I, Kpn I, Bgl II, Nsp V, and Nde I.

[0050] For ease of cloning, a NdeI restriction site was designed upstream of the start codon of the Sequence 1 (SEQ ID NO: 1) and a HindIII restriction site was designed near the terminator. The 480bps sequence was achieved by gene synthesis through chemical method. The sequence was double-digested with NdeI and HindIII and then inserted into the same double-digested expression vector pET-30a(+) to obtain a 5724bps recombinant plasmid, which was transformed into DH5α (TaKaRa, 9057) by heat shock method and cultured in LB medium containing 50 μg/mL kanamycin. Monoclones were selected and the correct transformants were screened by NdeI and HindIII double digestion. The correctness of the transformants was verified again by sequencing.

[0051] In another example, pET-28a(+), pET-23c(+) or pET-15b or the like can be used in place of the above pET-30a(+) vector.

[0052] The prokaryotic expression vector containing the protein sequence was transformed into *E. coli* BL21 (DE3), and the soluble human bFGF was induced to be expressed by adjusting the bacterial culture temperature, the induction temperature, the pH range, the glucose concentration, and the inducer concentration. The bacteria were collected by washing and filtering through hollow fiber; the fermented bacteria were disrupted by using high-pressure homogenization and maintaining at a low temperature; after adding an appropriate amount of nonionic surfactant, the supernatant was collected by low-temperature high-speed centrifugation, and the pellet was discarded.

[0053] Purification was carried out by use of a process such as weak cation exchange, heparin affinity chromatography or the like, and an appropriate amount of a protective agent such as mercaptoethanol, DTT or the like was added during the purification.

[0054] After the sample was purified by the above procedures, its purity was up to 95% and more. Experiments confirmed that about 600 mg of recombinant human bFGF protein could be prepared per 100 g of bacteria, and a significant high expression has been achieved.

2.2 Purity and molecular weight detection and sequencing

[0055] 15% SDS-PAGE electrophoresis showed that the human bFGF protein as obtained was a single band of approximately 18.5 KD (see Figure 2). The detection results of the high performance liquid chromatography with a C8 reverse phase column showed that the purity of the human bFGF as obtained in the present invention was more than 95% (see Figure 3).

[0056] "Ultra-high resolution, ultra-high accuracy, ultra-high sensitivity" Exactive Plus EMR was used for accurate molecular weight determination, and 6 components (components 1, 4, 5 ratios basically >10%, table 1) were detected in 4 batches of rh-bFGF stock solutions. Component 4 is the main component, and has an average molecular weight of 17121.02 Da; the inter-batch RSD% is 0.0007, with a deviation from the theoretical value of ≤41 ppm, and the relative ratio (calculated as peak intensity) is 70.7%-79.0%.

Table 1: HPLC-Exactive Plus EMR mass spectrometry for accurate molecular weight of test samples

Component	Batch	Experimental value (Da)	Theoretical value (Da)	Deviation (ppm)	Relative ratio (%)	Mean	RSD%
1	Stock Batch II	17049.21	17049.34Δ	8	5.2	17049.40	0.0019
	Stock Batch III	17049.77		25	12.6		
	Physical-chemical standard	17049.20		8	9.8		
4	Stock Batch I	17120.87	17120.42*	26	79.0	17121.02	0.0007
	Stock Batch II	17121.13		41	76.6		
	Stock Batch III	17120.98		33	70.7		
	Physical-chemical standard	17121.08		39	78.5		
5	Stock Batch I	17137.23	17136.42□	47	13.4	17137.34	0.0009
	Stock Batch II	17137.27		50	13.4		
	Stock Batch III	17137.51		64	10.6		

[0057] In addition, the recombinantly prepared human bFGF protein was analyzed for amino acid sequence, and it was confirmed that the amino acid sequence thereof was identical to that of native human bFGF (see Figure 5).

2.3 Biological activity assay

[0058] The samples were tested for *in vitro* activity using balb/c3T3 cells. Cell proliferation was judged by MTT assay. The results showed that the effect of promoting proliferation of balb/c3T3 cells by the obtained human bFGF was consistent with that by the bFGF active standard (NISCB) (see Figure 6).

Example 3. Preparation of pharmaceutical compositions

Pharmaceutical composition 1:

[0059]

recombinant human-basic fibroblast growth factor	2500-10000 IU;
human serum albumin	0.025-0.375 mg/mL;
thickener	5.0-15.0 mg/mL;
sodium chloride	5.0-12.5 mg/mL;
heparin sodium	0.25-5.0 μg/mL;
sodium dihydrogen phosphate	0.25-1.25 mg/mL;
disodium hydrogen phosphate	1.25-3.75 mg/mL.

1. The buffer system may be the above sodium dihydrogen phosphate and disodium hydrogen phosphate, or a boric acid-borax buffer system, a citric acid-disodium hydrogen phosphate buffer system or the like. Preferably, the pharmaceutical composition has a pH of from 6.5 to 7.5.

2. The thickener is polyvinyl alcohol, sodium hyaluronate, hypromellose, poloxamer, or the like, and preferably polyvinyl alcohol.

3. Preparation procedure:

[0060]

(1) Polyvinyl alcohol is dispersed and dissolved in an appropriate amount of water for injection, autoclaved at 121 °C for 30 min, cooled to room temperature, and ready for use;

(2) Recombinant human-basic fibroblast growth factor, human serum albumin, heparin sodium, sodium chloride, sodium dihydrogen phosphate, and disodium hydrogen phosphate are dissolved in an appropriate amount of water for injection, and sterile filtered through a 0.22 μm filter membrane;

(3)The solutions obtained in the step (1) and in the step (2) are uniformly mixed under a sterile condition, made up to the fixed volume with sterile water for injection, and thus obtained;

(4) The sterile solution is filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount is 0.4mL, and thus the finished product is obtained.

Pharmaceutical Composition 2:

[0061]

recombinant human-basic fibroblast growth factor	2500-10000 IU;
human serum albumin	0.1-0.375mg/mL;
heparin sodium	25.0-75.0 μg/mL;
carbomer	1.25-12.5 mg/mL;
neutralizing agent	1.25-12.5 mg/mL;

(continued)

glycerol	12.5-50 mg/mL.

1. The carbomer is a series of Carbomer 940, Carbomer 934, Carbomer 974, Carbomer 980, etc., preferably a Carbomer 980 series;
2. The neutralizing agent is sodium hydroxide, potassium hydroxide, potassium hydrogen carbonate, borax and triethanolamine, preferably triethanolamine.

3. Preparation procedure:

[0062]

(1) Carbomer is dispersed in an appropriate amount of water for injection, stirred uniformly, and then swelled overnight, and ready for use;

(2) Triethanolamine is added to the carbomer dispersion, stirred into a transparent uniform gel base, autoclaved at 121 °C for 30 min, cooled to room temperature after sterilization is completed, and ready for use;

(3) Glycerol, human serum albumin, heparin sodium, and recombinant human-basic fibroblast growth factor are added into an appropriate amount of room temperature water for injection, stirred uniformly, and then passed through a 0.22 μm filter membrane under a sterile condition, mixed with the gel base in the step (2), and then quantified and stirred uniformly;

(4) The sterile gel is filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount is 0.4g, and thus the finished product is obtained.

Pharmaceutical Composition 3:

[0063]

recombinant human-basic fibroblast growth factor	1000-9000 IU
human serum albumin	0.01-5.0 mg/mL;
thickener	0.1-20.0mg/mL;
sodium dihydrogen phosphate	0.2-2.5 mg/mL;
disodium hydrogen phosphate	0.5-5.0 mg/mL;
sodium chloride	1.0-5.0 mg/mL;
humectant	0.1-50.0 mg/mL.

1. The buffer salt system may be the above sodium dihydrogen phosphate and disodium hydrogen phosphate, or a boric acid-borax buffer system, a citric acid-disodium hydrogen phosphate buffer system or the like; preferably, the pH is from 6.5 to 7.5.

2. The thickener is polyvinyl alcohol, sodium hyaluronate, hypromellose, poloxamer, or the like, and preferably polyvinyl alcohol. The humectant is glycerin, propylene glycol or a mixture thereof.

3. Preparation procedure:

[0064]

(1) The thickener and sodium chloride are dispersed and dissolved in an appropriate amount of water for injection, and autoclaved at 121 °C for 30 min;

(2) Recombinant human-basic fibroblast growth factor, human serum albumin, humectant, sodium dihydrogen phosphate, and disodium hydrogen phosphate are dissolved in an appropriate amount of water for injection;

(3) The agent solution in the step (2) is filtered through a 0.22 μm microporous filter membrane and then mixed with

the agent solution obtained in the step (1), and made up to 1 mL with water for injection;

(4) The agent solution obtained in the step (3) is filled into a packaging container containing no bacteriostatic agent, and the volume of the container is in the range of 0.4 g/piece, and thus, the finished product is obtained.

[0065] In addition, the following pharmaceutical compositions (see Table 2) were also prepared. Among them, the eye drops were prepared as 100 ml, and the external gels were prepared as 100 g.

Table 2: Pharmaceutical compositions of Preparation Examples 1-10

	Preparation Example 1	Preparation Example 2	Preparation Example 3	Preparation Example 4	Preparation Example 5	Preparation Example 6	Preparation Example 7	Preparation Example 8	Preparation Example 9	Preparation Example 10
polyvinyl alcohol	10.0mg/mL	10.0mg/mL	10.0mg/mL	10.0mg/mL	10.0mg/mL	10.0mg/mL				
Carbomer 940							8.0mg/mL	6.0mg/mL	6.0mg/mL	5.0mg/mL
triethanolamine							6.0mg/mL	5.0mg/mL	5.0mg/mL	5.0mg/mL
rh-bFGF	5000IU/mL	5000IU/mL	4200IU/mL	4200IU/mL	4200IU/mL	5000IU/mL	4500IU/mL	4200IU/mL	4500IU/mL	4200IU/mL
glycerol							25.0mg/mL	25.0mg/mL	25.0mg/mL	25.0mg/mL
human serum albumin	0.25mg/mL	0.20mg/mL	0.20mg/mL	0.10mg/mL	0.20mg/mL	0.25mg/mL	0.30mg/mL	0.30mg/mL	0.30mg/mL	0.20mg/mL
heparin sodium	25 μg/mL	20 μg/mL	20 μg/mL	10 μg/mL	20 μg/mL	25 μg/mL	70 μg/mL	70 μg/mL	70 μg/mL	50 μg/mL
sodium chloride	8.0mg/mL	8.0mg/mL	8.0mg/mL	8.0mg/mL	8.0mg/mL	8.0mg/mL				
citric acid					3.706mg/mL	3.706mg/mL				
sodium dihydrogen phosphate	0.425mg/mL	0.425mg/mL	0.425mg/mL	0.425mg/mL						
disodium hydrogen phosphate	2.50mg/mL	2.50mg/mL	2.50mg/mL	2.50mg/mL	590mg/mL	590mg/mL				

Preparation Example 1:

[0066]

(1) 1.0 g of polyvinyl alcohol was dispersed and dissolved in an appropriate amount of water for injection, autoclaved, cooled to room temperature, and ready for use;

(2) 500000 IU of recombinant human-basic fibroblast growth factor, 25mg of human serum albumin, 2.5mg of heparin sodium, 800mg of sodium chloride, 42.5mg of sodium dihydrogen phosphate, and 250mg of disodium hydrogen phosphate were dissolved in an appropriate amount of water for injection, and sterile filtered through a 0.22 μm filter membrane;

(3)The solutions obtained in the step (1) and in the step (2) were uniformly mixed under a sterile condition, made up to 100 mL with sterile water for injection, and thus obtained;

(4) The sterile solution was filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount was 0.4mL, and thus the finished product was obtained.

Preparation Example 2:

[0067]

(1) 0.5 g of polyvinyl alcohol was dispersed and dissolved in an appropriate amount of water for injection, autoclaved, cooled to room temperature, and ready for use;

(2) 500000 IU of recombinant human-basic fibroblast growth factor, 20mg of human serum albumin, 2.0mg of heparin sodium, 800mg of sodium chloride, 42.5mg of sodium dihydrogen phosphate, and 250mg of disodium hydrogen phosphate were dissolved in an appropriate amount of water for injection, and sterile filtered through a 0.22 μm filter membrane;

(3)The solutions obtained in the step (1) and in the step (2) were uniformly mixed under a sterile condition, made up to 100 mL with sterile water for injection, and thus obtained;

(4) The sterile solution was filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount was 0.4mL, and thus the finished product was obtained.

Preparation Example 3:

[0068]

(1) 1.0 g of polyvinyl alcohol was dispersed and dissolved in an appropriate amount of water for injection, autoclaved, cooled to room temperature, and ready for use;

(2) 420000 IU of recombinant human-basic fibroblast growth factor, 20mg of human serum albumin, 2.0mg of heparin sodium, 800mg of sodium chloride, 42.5mg of sodium dihydrogen phosphate, and 250mg of disodium hydrogen phosphate were dissolved in an appropriate amount of water for injection, and sterile filtered through a 0.22 μm filter membrane;

(3)The solutions obtained in the step (1) and in the step (2) were uniformly mixed under a sterile condition, made up to 100 mL with sterile water for injection, and thus obtained;

(4) The sterile solution was filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount was 0.4mL, and thus the finished product was obtained.

Preparation Example 4:

[0069]

(1) 1.5 g of polyvinyl alcohol was dispersed and dissolved in an appropriate amount of water for injection, autoclaved, cooled to room temperature, and ready for use;

(2) 420000 IU of recombinant human-basic fibroblast growth factor, 10mg of human serum albumin, 1.0mg of heparin sodium, 800mg of sodium chloride, 42.5mg of sodium dihydrogen phosphate, and 250mg of disodium hydrogen phosphate were dissolved in an appropriate amount of water for injection, and sterile filtered through a 0.22 μm filter membrane;

(3)The solutions obtained in the step (1) and in the step (2) were uniformly mixed under a sterile condition, made up to 100 mL with sterile water for injection, and thus obtained;

(4) The sterile solution was filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount was 0.4mL, and thus the finished product was obtained.

Preparation Example 5:

[0070]

(1) 1.0 g of polyvinyl alcohol was dispersed and dissolved in an appropriate amount of water for injection, autoclaved, cooled to room temperature, and ready for use;

(2) 420000 IU of recombinant human-basic fibroblast growth factor, 20mg of human serum albumin, 2.0mg of heparin sodium, 800mg of sodium chloride, 370.6mg of citric acid, and 5.9g of disodium hydrogen phosphate were dissolved in an appropriate amount of water for injection, and sterile filtered through a 0.22 μm filter membrane;

(3)The solutions obtained in the step (1) and in the step (2) were uniformly mixed under a sterile condition, made up to 100 mL with sterile water for injection, and thus obtained;

(4) The sterile solution was filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount was 0.4mL, and thus the finished product was obtained.

Preparation Example 6:

[0071]

(1) 1.0 g of polyvinyl alcohol was dispersed and dissolved in an appropriate amount of water for injection, autoclaved, cooled to room temperature, and ready for use;

(2) 500000 IU of recombinant human-basic fibroblast growth factor, 25mg of human serum albumin, 2.5mg of heparin sodium, 800mg of sodium chloride, 370.6mg of citric acid, and 5.9g of disodium hydrogen phosphate were dissolved in an appropriate amount of water for injection, and sterile filtered through a 0.22 μm filter membrane;

(3)The solutions obtained in the step (1) and in the step (2) were uniformly mixed under a sterile condition, made up to 100 mL with sterile water for injection, and thus obtained;

(4) The sterile solution was filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount was 0.4mL, and thus the finished product was obtained.

Preparation Example 7:

[0072]

(1) 0.80g of Carbomer 940 was weighted and dispersed in an appropriate amount of room temperature water for injection, stirred for 60-120 min, swelled overnight, and ready for use;

(2) 0.60g of triethanolamine was added to the Carbomer 940 dispersion, stirred into a transparent uniform gel base, and then subjected to moist heat sterilization (121 °C, 30 min), cooled to room temperature after sterilization was completed, and ready for use;

(3) 2.50g of glycerol, 30.0mg of human serum albumin, 7.0mg of heparin sodium, and 450000 IU of recombinant human-basic fibroblast growth factor were added into an appropriate amount of room temperature water for injection, stirred uniformly, and then passed through a 0.22 μm filter membrane under a sterile condition, mixed with the gel base in the step (2), and then quantified and stirred uniformly;

(4) The sterile gel was filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount was 0.4g, and thus the finished product was obtained.

Preparation Example 8:

[0073]

(1) 0.60g of Carbomer 940 was weighted and dispersed in an appropriate amount of room temperature water for injection, stirred for 60-120 min, swelled overnight, and ready for use;

(2) 0.50g of triethanolamine was added to the Carbomer 940 dispersion, stirred into a transparent uniform gel base, and then subjected to moist heat sterilization (121 °C, 30 min), cooled to room temperature after sterilization was completed, and ready for use;

(3) 2.50g of glycerol, 30.0mg of human serum albumin, 7.0mg of heparin sodium, and 420000 IU of recombinant human-basic fibroblast growth factor were added into an appropriate amount of room temperature water for injection, stirred uniformly, and then passed through a 0.22 μm filter membrane under a sterile condition, mixed with the gel base in the step (2), and then quantified and stirred uniformly;

(4) The sterile gel was filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount was 0.4g, and thus the finished product was obtained.

Preparation Example 9:

[0074]

(1) 0.60g of Carbomer 974 was weighted and dispersed in an appropriate amount of room temperature water for injection, stirred for 60-120 min, swelled overnight, and ready for use;

(2) 0.50g of triethanolamine was added to the Carbomer 974 dispersion, stirred into a transparent uniform gel base, and then subjected to moist heat sterilization (121 °C, 30 min), cooled to room temperature after sterilization was completed, and ready for use;

(3) 2.50g of glycerol, 30.0mg of human serum albumin, 7.0mg of heparin sodium, and 450000 IU of recombinant human-basic fibroblast growth factor were added into an appropriate amount of room temperature water for injection, stirred uniformly, and then passed through a 0.22 μm filter membrane under a sterile condition, mixed with the gel base in the step (2), and then quantified and stirred uniformly;

(4) The sterile gel was filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount was 0.4g, and thus the finished product was obtained.

Preparation Example 10:

[0075]

(1) 0.50g of Carbomer 974 was weighted and dispersed in an appropriate amount of room temperature water for injection, stirred for 60-120 min, swelled overnight, and ready for use;

(2) 0.50g of triethanolamine was added to the Carbomer 974 dispersion, stirred into a transparent uniform gel base, and then subjected to moist heat sterilization (121 °C, 30 min), cooled to room temperature after sterilization was completed, and ready for use;

(3) 2.50g of glycerol, 20.0mg of human serum albumin, 5.0mg of heparin sodium, and 420000 IU of recombinant

human-basic fibroblast growth factor were added into an appropriate amount of room temperature water for injection, stirred uniformly, and then passed through a 0.22 μm filter membrane under a sterile condition, mixed with the gel base in the step (2), and then quantified and stirred uniformly;

(4) The sterile gel was filled by using a three-in-one filling machine of blowing, filling and sealing, and the filling amount was 0.4g, and thus the finished product was obtained.

Example 4. Stability study

1) Study of the effect of amino acids on the stability of the rh-bFGF stock solution

[0076] The rh-bFGF stock solution itself is unstable in nature, and tends to polymerize and thus precipitate at room temperature. Therefore, different stabilizers were selected and used, and primarily screened for the stability of the stock solution. 5% and 2% mannitol, 5% and 2% glycine, and 2% dextran were selected and used, and allowed to stand at 25 °Cenvironment for 17 days. Protein concentrations were measured on days 0, 7, and 17, respectively, and the results are shown in Table 3.

Table 3: Rate of change in protein concentration of the rh-bFGF stock solution in the screening test of stabilizers

Sample	Day 0	Day 7	Day 17
No stabilizer	100.00%	29.96%	13.63%
5% mannitol	100.00%	49.80%	17.31%
2% mannitol	100.00%	53.29%	15.66%
5% glycine	100.00%	95.67%	86.26%
2% glycine	100.00%	94.74%	82.73%
2% dextran	100.00%	51.65%	16.20%

[0077] The results showed that 5% glycine was effective in preventing protein from precipitation. There remained 86.26% protein after being placed under the thermal destructive condition for 17 days. After 17 days, the effect of glycine was significantly better than that of mannitol and dextran, while only 13.6% of the protein was left with no stabilizer added.

2) Study of the effect of histidine on the stability of the rh-bFGF stock solution

[0078] Different amino acids (glycine, histidine, arginine) and Tween 20 were selected and used, and further screened for the stability of the stock solution, and allowed to stand at 25 °Cenvironment for 18 h. The results are shown in Figure 7.

[0079] The results showed that 3% histidine and 0.03% Tween 20 were more effective than 5% glycine with respect to the stability of the rh-bFGF stock solution. The purity of the protein could still be maintained above 85% after being placed under the thermal destructive condition for 18 h. Histidine and Tween 20 were superior to glycine in protecting rh-bFGF.

3) **Study of the effect of HSA on the stability of rh-bFGF stock solution**

[0080] Since HSA would interfere with the determination of protein content, the stock solutions with or without HSA were placed at 25 °Cenvironment for 18 h, and change in the purity of the protein after being subjected to heat-damage was detected by high-resolution chromatography. The results are shown in Figure 8.

[0081] The results showed that HSA could significantly improve the stability of rh-bFGF. With a change rate of 95% purity as an indicator, samples containing no HSA can only be maintained for 4 h under the stress condition, and can be extended to 15 hours after addition of HSA. It has been shown that HSA is an excellent protein protectant for bFGF.

Example 5. Study on the efficacy in an animal dry eye model

[0082] In this study, New Zealand rabbit dry eye model was selected and used, the clinical indicators (tear secretion, and tear film break-up time) of the model were observed, and the clinical treatment effect of the medicament for dry eye was evaluated. New Zealand rabbits were divided into negative control group (Negative control, untreated), model control

group (Model, treated with alkali burn but no any eye drops were added), treatment group with sodium hyaluronate eye drop (HA group), and the Group D (4.0 μg/mL), E (8.0 μg/mL), F (16.0 μg/mL), and G (32.0 μg/mL) which were divided according to the concentration of the rh-bFGF eye drops of Preparation Example 3 as used. The negative control group had 72 rabbits/group, and the remaining groups had 8 rabbits/group. The dosage was 300 μl/eye/day.

(1) Method for measuring the amount of tear secretion (both eyes) (the wet length of phenol red cotton thread):

[0083] The secretion of tears from New Zealand rabbits was measured using the Schirmer I test. That is, the phenol red cotton thread was clamped with ophthalmic forceps, and placed in the outer canthus of the New Zealand rabbit. After 60 s, the phenol red cotton thread was taken out and measured for the wet length. The phenol red cotton thread turned red after being wet, and the eye wetness was determined according to the wet length. The experimental results are shown in Figure 9.

[0084] As shown in Figure 9, the wet lengths of the phenol red cotton thread in the model control group all were significantly decreased as compared with those in the negative control eyes. On day 10 of administration, the wet lengths of the phenol red cotton thread in the rh-bFGF eye drops D, E, F, and G groups all were significantly longer with statistically significant differences, as compared with the model control group.

(2) Method for measuring tear film break-up time (both eyes):

[0085] 2μl of 0.5% sodium fluorescein solution was instilled into the lower eyelid conjunctival sac of New Zealand rabbit eyes using an adjustable pipette. After several times of manual blinking with constant force, the rabbit eyes were opened with a constant force and the cornea was observed with a slit lamp microscope and cobalt blue light. When a black area appears in the corneal green film, the tear film is indicated to be broken. Three measurements were taken continuously and the average value was taken. Less than 10 seconds of the tear film break-up time indicates that the tear film is unstable, which is a prominent marker of KCS caused by the lack of mucin in tears, suggesting that the goblet cells of the conjunctiva are seriously damaged or lost, and it is easy to cause dry eye. The experimental results are shown in Figure 10.

[0086] As shown in Figure 10, the tear film break-up times of the model control group all were significantly shorter than those of the negative control eyes on day 10 of administration, as compared to the negative control eyes. On day 10 of administration, the tear film break-up time of the rh-bFGF eye drops D, E, F, and G groups all were significantly prolonged with statistically significant differences, as compared with the model control group.

[0087] In summary, the results of the phenol red cotton thread test of the tear secretion showed that the eye drops of the present invention could improve the tear secretion amount of the model animals (see Figure 9); the tear film break-up time test showed that there was a significant improvement in the tear film break-up time in the dry eye model (see Figure 10). With an increase in dosage, rh-bFGF eye drops had a significant improvement on the dry eye model of alkal burned New Zealand rabbits.

[0088] Various modifications, substitutions, changes and equivalents will occur to those skilled in the art, although some features of the present invention has been set forth and illustrated herein. Therefore, it is to be understood that the appended claims are intended to cover all such modifications and changes that fall into the true spirit and scope of the present invention.

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1

Claims

1. A mutated nucleic acid molecule encoding a recombinant human-basic fibroblast growth factor, which comprises a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3, or comprises a sequence having at least 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

2. The mutated nucleic acid molecule of claim 1, the sequence of which is set forth in SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

3. A recombinant human-basic fibroblast growth factor encoded by the mutated nucleic acid molecule of claim 1 or 2.

4. A pharmaceutical composition comprising the human-basic fibroblast growth factor of claim 3 and at least one pharmaceutically acceptable excipient comprising at least one stabilizer selected from the group consisting of glycine, histidine, arginine, Tween, heparin sodium or human serum albumin (HSA).

5. The pharmaceutical composition of claim 4 wherein said stabilizer is glycine.

6. The pharmaceutical composition of claim 4, wherein the stabilizer is histidine.

7. The pharmaceutical composition of claims 4-6, wherein the excipient further comprises a buffer system selected from the group consisting of sodium dihydrogen phosphate-disodium hydrogen phosphate, citric acid-disodium hydrogen phosphate or boric acid-borax.

8. The pharmaceutical composition of claims 4-7, wherein the excipient further comprises a thickener selected from the group consisting of polyvinyl alcohol, sodium hyaluronate, methyl cellulose, hydroxymethyl cellulose, hydroxyethyl cellulose, hypromellose, poloxamer or carbomer.

9. The pharmaceutical composition of any one of claims 4-8, wherein the excipient further comprises a neutralizing agent selected from the group consisting of triethanolamine, sodium hydroxide, potassium hydroxide, sodium carbonate, sodium hydrogen carbonate, potassium hydrogen carbonate or borax.

10. The pharmaceutical composition of any one of claims 4-9, wherein the excipient further comprises a humectant selected from the group consisting of glycerin, propylene glycol or a mixture thereof.

11. The pharmaceutical composition of any one of claims 4-10 which is buffered to a pH of from 6.0 to 8.0.

12. The pharmaceutical composition of any one of claims 4-11, which is an ophthalmic composition, preferably in the form of an eye drop or a gel.

13. The pharmaceutical composition of claim 4 comprising:

recombinant human-basic fibroblast growth factor	2500-10000 IU;
human serum albumin	0.025-0.375 mg/mL;
thickener	5.0-15.0 mg/mL;
sodium chloride	5.0-12.5 mg/mL;
heparin sodium	0.25-5.0 μg/mL;
sodium dihydrogen phosphate	0.25-1.25 mg/mL;
disodium hydrogen phosphate	1.25-3.75 mg/mL.

14. The pharmaceutical composition of claim 4 comprising:

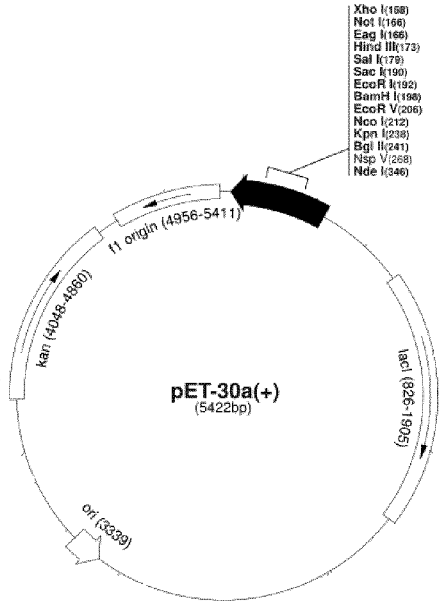
recombinant human-basic fibroblast growth factor	2500-10000 IU;
human serum albumin	0.1-0.375mg/mL;
heparin sodium	25.0-75.0 μg/mL;
carbomer	1.25-12.5 mg/mL;
neutralizing agent	1.25-12.5 mg/mL;
glycerol	12.5-50 mg/mL.

15. The pharmaceutical composition of claim 4 comprising:

recombinant human-basic fibroblast growth factor	1000-9000 IU
human serum albumin	0.01-5.0 mg/mL;
thickener	0.1-20.0mg/mL;
sodium dihydrogen phosphate	0.2-2.5 mg/mL;
disodium hydrogen phosphate	0.5-5.0 mg/mL;
sodium chloride	1.0-5.0 mg/mL;
humectant	0.1-50.0 mg/mL.

16. A method of treating dry eye, the method comprising administering to a patient in need thereof a therapeutically effective amount of the pharmaceutical composition of any one of claims 4-15.

17. A method for preparing the pharmaceutical composition of any one of claims 4-15, which comprises mixing the human-basic fibroblast growth factor with at least one pharmaceutically acceptable excipient.



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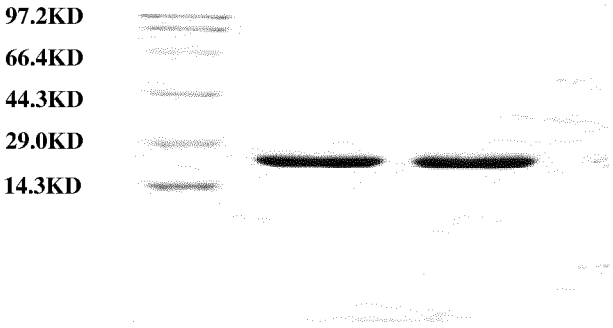

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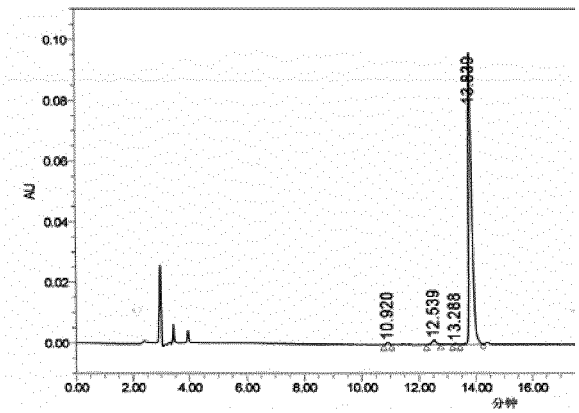
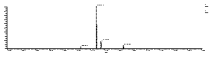
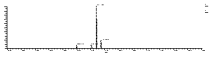


Figure 3

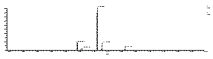
bFGF-0911



bFGF-0912



bFGF-0913



bFGF-1001

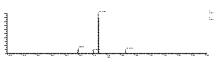


Figure 4

atg gca gcc ggg agc atc acc acg ctg ccc gcc ttg ccc gag gat ggc ggc agc ggc gcc ttc ccg ccc ggc cac ttc aag gac ccc aag cgg ctg tac tgc aaa aac ggg ggc ttc ttc ctg cgc atc cac ccc gac ggc cga gtt gac ggg gtc cgg gag aag agc gac cct cac atc aag cta caa ctt caa gca gaa gag aga gga gtt gtg tct atc aaa gga gtg tgt gct aac cgt tac ctg gct atg aag gaa gat gga aga tta ctg gct tct aaa tgt gtt acg gat gag tgt ttc ttt ttt gaa cga ttg gaa tct aat aac tac aat act tac cgg tca agg aaa tac acc agt tgg tat gtg gca ctg aaa cga act ggg cag tat aaa ctt gga tcc aaa aca gga cct ggg cag aaa gct ata ctt ttt ctt cca atg tct gct aag agc tga

Native human bFGF cDNA sequence (SEQ ID NO:4)

1	maagsittlp	alpedggsga	fppghfkdpk	rlyckнggff	lrihpdgrvd	gvreksdphi
61	klqlqaeerg	vvsikgvcan	rylamkedgr	llaskcvtde	cffferlesn	nyntyrsrky
121	tswyvalkrt	gqyklgsktg	pgqkailflp	msaks		

Amino acid sequence of native human bFGF (SEQ ID NO:5)

1	maagsittlp	alpedggsga	fppghfkdpk	rlyckнggff	lrihpdgrvd	gvreksdphi
61	klqlqaeerg	vvsikgvcan	rylamkedgr	llaskcvtde	cffferlesn	nyntyrsrky
121	tswyvalkrt	gqyklgsktg	pgqkailflp	msaks		

Mutated human bFGF amino acid sequence (SEQ ID NO:6)

Figure 5

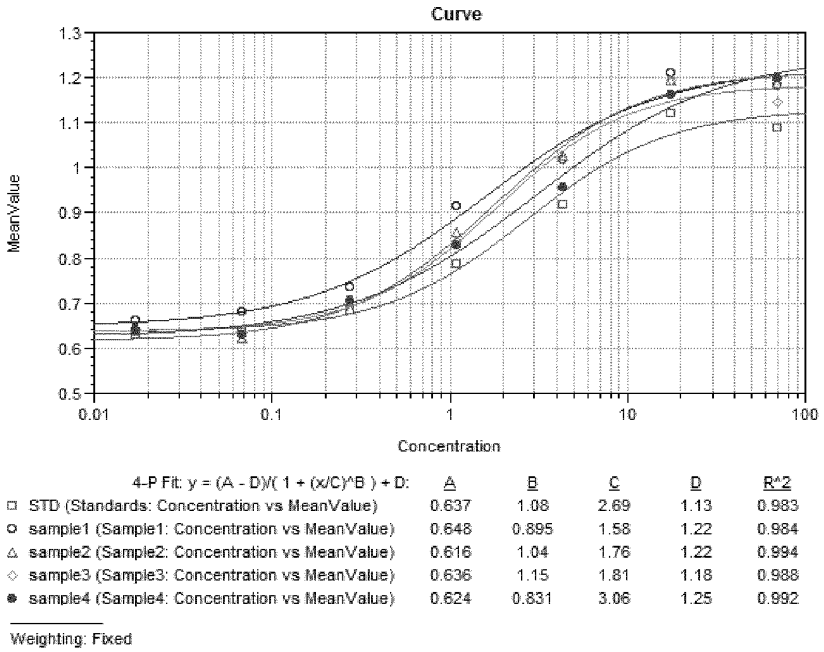


Figure 6

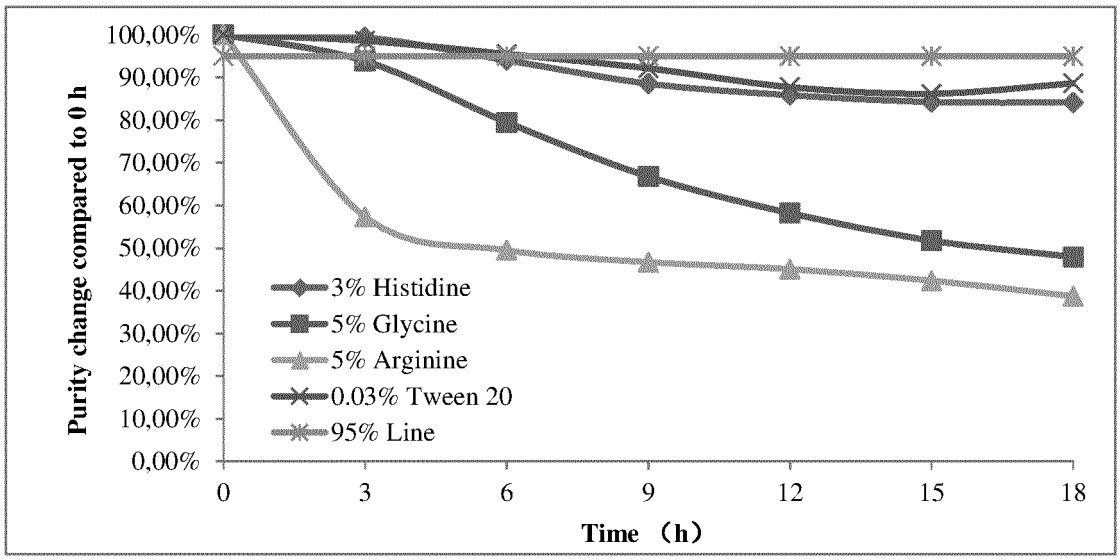


Figure 7

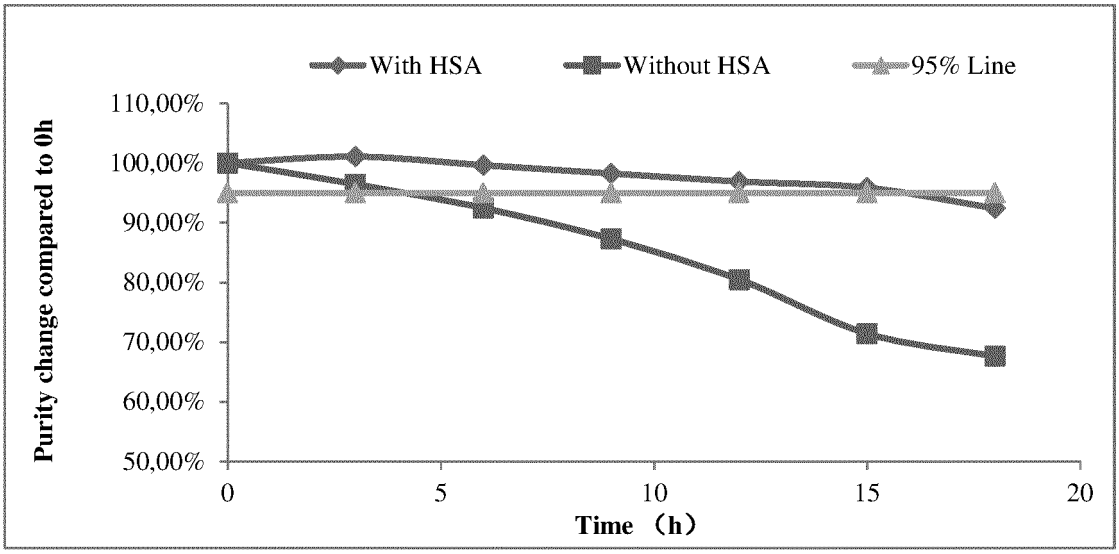


Figure 8

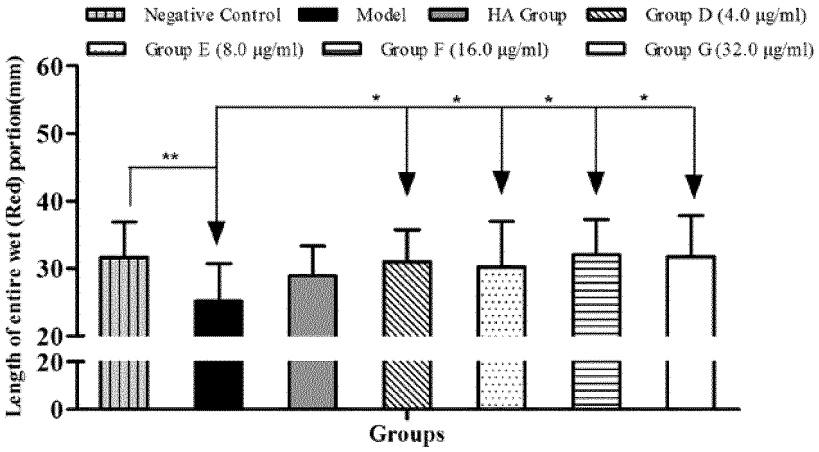


Figure 9

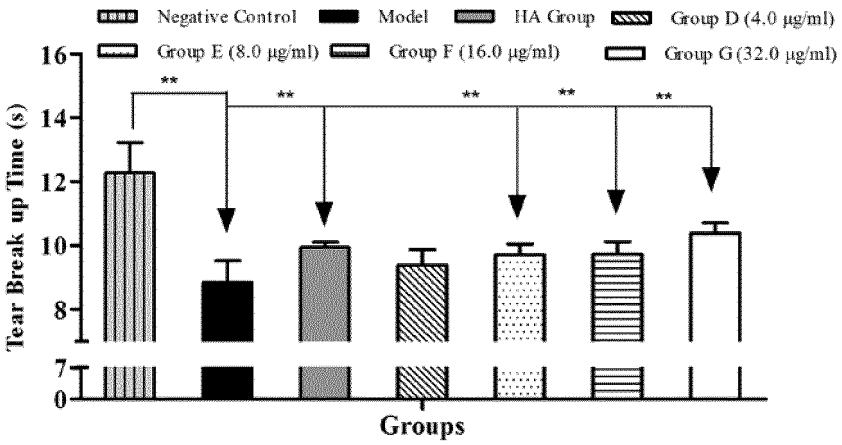


Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2017/089823

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/12 (2006.01) i; C12N 15/70 (2006.01) i; A61K 38/18 (2006.01) i; A61P 27/02 (2006.01) i
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N, A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Data bases: CNABS, CNKI, DWPI, SIPOABS, CNTXT, WOTXT, USTXT, EPTXT, ISI Web of Knowledge, google, EMBL+GenBank+ddbj, NATIONAL BIO-SEQUENCE DATABASE OF CHINESE PATENT;
Keywords: 重组人碱性成纤维细胞生长因子, 大肠杆菌, 表达, 稳定剂, 甘氨酸, 组氨酸, 缓冲液, 缓冲体系, 增稠剂, 中和剂, 保湿剂, 滴眼液, 凝胶, 干眼症, recombinant, humanbasic fibroblast growth factor, rh-bFGF, dry eye, stabilizer, moisturizer, eye drops, neutralizer, e. coli, search for SEQ ID NO: 1-6

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 1448510 A (BEIJING TRI-PRIME GENETIC ENGINEERING CO., LTD.), 15 October 2003 (15.10.2003), claims 1-3, and description, page 2, paragraph 3 and sequence table	1-3
X	CN 104984326 A (ZHUHAI ESSEX BIO-PHARMACEUTICAL COMPANY LIMITED), 21 October 2015 (21.10.2015), abstract, claims 1-6, and description, paragraphs 5-13 and embodiments 1-4	4-17
Y	CN 1448510 A (BEIJING TRI-PRIME GENETIC ENGINEERING CO., LTD.), 15 October 2003 (15.10.2003), claims 1-3, and description, page 2, paragraph 3 and sequence table	4-17
Y	CN 104606666 A (ZHUHAI ESSEX BIO-PHARMACEUTICAL COMPANY LIMITED), 13 May 2015 (13.05.2015), abstract, claims 1-2, and description, paragraph 3	4-17
A	CN 104984326 A (ZHUHAI ESSEX BIO-PHARMACEUTICAL COMPANY LIMITED), 21 October 2015 (21.10.2015), abstract, claims 1-6, and description, paragraphs 5-13 and embodiments 1-4	1-3

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 March 2018	22 March 2018
Name and mailing address of the ISA State Intellectual Property Office of the P. R. China No. 6, Xitucheng Road, Jimenqiao Haidian District, Beijing 100088, China Facsimile No. (86-10) 62019451	Authorized officer LUO, Xiao Telephone No. (86-10) 62089158

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2017/089823

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2017/089823

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 16
because they relate to subject matter not required to be searched by this Authority, namely:
[1] claim 16 relates to a method for the treatment of a living human or animal body, which falls within the cases, set out in PCT Rule 39.1(iv), for which an international search is not required, and the search and the written opinion therefor are provided on the basis that said claim is reasonably expected to be "a usage for the preparation of a pharmaceutical composition for the treatment of xerophthalmia".

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2017/089823

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
CN 1448510 A	15 October 2003	None	
CN 104984326 A	21 October 2015	None	
CN 104606666 A	13 May 2015	None	

Form PCT/ISA/210 (patent family annex) (July 2009)